Vectorial Ca^{2+} flux from the extracellular space to the endoplasmic reticulum via a restricted cytoplasmic compartment regulates inositol 1,4,5-trisphosphate-stimulated Ca^{2+} release from internal stores in vascular endothelial cells

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Depletion of the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} store of vascular endothelial cells after selective inhibition of the endoplasmic-reticulum (ER) Ca^{2+} pump by thapsigargin or 2,5-di-t-butylhydroquinone (BHQ) increases Ca^{2+} influx from the extracellular space in the absence of phosphoinositide hydrolysis. One model to account for these results suggests a close association between the internal store and the plasmalemma, allowing for the vectorial movement of Ca^{2+} from the extracellular space to the ER. Furthermore, recent evidence suggests that Ins(1,4,5)P_3-induced Ca^{2+} release from intracellular stores is regulated by the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Thus agonist-induced Ca^{2+} entry may directly regulate Ca^{2+} release from internal stores. To test these hypotheses, we examined the effect of 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole (SKF 96365), an inhibitor of Ca^{2+} influx, on unidirectional $^{45}Ca^{2+}$ efflux (i.e. retrograde radioisotope flux via the influx pathway) and on $[Ca^{2+}]_i$ as measured by fura-2. Bradykinin produced a transient increase in $[Ca^{2+}]_i$, reflecting release of Ca^{2+} from internal stores, and a sustained increase indicative of Ca^{2+} influx. In the absence of agonist, $^{45}Ca^{2+}$ efflux was slow and monoexponential with time. Addition of BK dramatically increased $^{45}Ca^{2+}$ efflux; 50–60% of the $^{45}Ca^{2+}$ associated with the cell monolayer was released within 2 min after addition of bradykinin. Both the bradykinin-induced change in $[Ca^{2+}]_i$ and the stimulation of $^{45}Ca^{2+}$ efflux was completely blocked by loading the cells with the Ca^{2+} chelator BAPTA. At a supermaximal concentration of bradykinin (50 nM), SKF 96365 (50 μM) inhibited the rise in $[Ca^{2+}]_i$ attributed to influx without affecting release from internal stores. At a threshold concentration of bradykinin (2 nM), SKF 96365 blocked influx, but stimulated Ca^{2+} release from internal stores, as indicated by increases in both the transient component of the fura-2 response and $^{45}Ca^{2+}$ efflux. Thapsigargin (200 nM) and BHQ (10 μM) produced an increase in $^{45}Ca^{2+}$ efflux that was completely blocked by SKF 96365 or by cytosolic loading with BAPTA. These results suggest the existence of a restricted sub-plasmalemmal space that is defined by an area of surface membrane which contains the Ca^{2+}-influx pathway but is devoid of Ca^{2+} pumps, and by a section of ER that is rich in thapsigargin-sensitive Ca^{2+}-pump units. Blockade of Ca^{2+} influx through the agonist-activated pathway by SKF 96365 increases Ca^{2+} release from internal stores, and suggests that a rise in $[Ca^{2+}]_i$ within the restricted space as a direct result of influx inhibits either Ins(1,4,5)P_3 binding or effect at the Ca^{2+}-release channel. A model is proposed in which these structural features provide for vectorial Ca^{2+} flux through the cell.

INTRODUCTION

It is now well established in vascular endothelial cells, and in a variety of non-excitable cell types, that specific receptor stimulation results in a biphasic increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$); an initial transient component reflects the release of Ca^{2+} from internal stores, whereas a more sustained elevation reflects the influx of Ca^{2+} from the extracellular space (Colden-Stanfield et al., 1987; Schilling et al. 1989). Although both the release of Ca^{2+} from internal storage sites and the influx of Ca^{2+} from the extracellular space normally require the production of Ins(1,4,5)P_3, studies using the Ca^{2+}-ATPase inhibitors thapsigargin, cyclopiazonic acid or 2,5-di-t-butylhydroquinone (BHQ) indicate that depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store can stimulate Ca^{2+} influx in the absence of measurable phosphoinositide hydrolysis (Schilling et al., 1992). These results support the hypothesis that Ca^{2+} influx is related to the level of Ca^{2+} within the internal store, i.e. the so-called capacitative Ca^{2+}-entry model (Putney, 1986, 1990, 1992).

Several lines of evidence suggest that the pathway activated by depletion of the Ins(1,4,5)P_3-sensitive internal store is indistinguishable from the agonist-activated influx pathway (Schilling et al., 1992). Both pathways allow Ca^{2+} and Ba^{2+} to enter the cell, and both exhibit similar sensitivity to La^{3+}, membrane potential and extracellular pH. Furthermore, both pathways are inhibited by 1-{β-[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl}-1*H*-imidazole (SKF 96365), a novel organic compound which has been shown to block agonist-activated Ca^{2+} influx in a variety of cells without affecting Ca^{2+} release from internal storage sites (Merritt et al., 1990; Chan and Greenberg, 1991; Graier et al., 1992; Schilling et al.,1992).

The molecular mechanism by which the repletion status of the internal store controls the activity of the plasmalemmal influx pathway has not been identified. Although it is clear that Ca^{2+} can be sequestered from the cytoplasm into the endoplasmic reticulum (ER) via the action of a Ca^{2+}-ATPase/pump, a number of studies suggest that internal Ca^{2+} stores can be replenished with Ca^{2+} directly from the extracellular space without a rise in

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; BHQ, 2,5-di-t-butylhydroquinone; ER, endoplasmic reticulum; HBS, Hepes-buffered saline; AM, acetoxymethyl ester; BAPTA, bis-(o-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; CPAE, calf pulmonary-artery endothelial cells; DMEM, Dulbecco's Modified Eagle's Medium.

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$[Ca^{2+}]_i$ above the normal basal value (Merritt and Rink, 1987; Schilling et al., 1988). These results are consistent with an early version of the capacitative model wherein direct communication between the extracellular space and the internal storage site allowed for the vectorial movement of Ca^{2+} into the ER before entry into the cytosolic compartment via Ins(1,4,5)P_3-activated channels (Putney, 1986; Merritt and Rink, 1987). In the absence of receptor stimulation and Ins(1,4,5)P_3 production, the internal stores would refill with Ca^{2+} directly with the extracellular space. This version of the model was initially attractive in that the level of Ca^{2+} within the ER could directly modulate Ca^{2+} influx. Recent studies, however, cast some doubt on this model. In isolated fura-2-loaded parotid acinar cells, addition of methacholine several minutes after thapsigargin caused a small transient increase in $[Ca^{2+}]_i$, but did not appear to alter the sustained component of the $[Ca^{2+}]_i$ response (Takemura et al., 1989). It is argued that if thapsigargin-initiated depletion of the internal store activates a direct pathway for Ca^{2+} movement into the ER, then subsequent stimulation with an Ins(1,4,5)P_3-dependent agonist, in this case methacholine, should enhance influx. In vascular endothelial cells the result is not as clear. An elevation of the steady-state component could be seen after addition of bradykinin at various times after either thapsigargin or cyclopiazonic acid (Schilling et al., 1992). Although this may be related to a time-dependent depletion of the agonist-sensitive Ca^{2+} pool, it is possible that an important component of Ca^{2+} influx into the ER may bypass the cytoplasm. Furthermore, studies investigating the characteristics of ER Ca^{2+} sequestration in permeabilized cells and subsequent release by GTP and Ins(1,4,5)P_3 suggest that GTP hydrolysis may be linked to a transmembrane conveyance of Ca^{2+} from one intracellular pool to another and that similar junction formation may occur between the ER and the plasmalemma (Mullaney et al., 1987, 1988; Ghosh et al., 1989; Gill et al., 1992). Such junction formation may be transient and regulated by complex biochemical mechanisms.

Irrespective of the exact path taken by Ca^{2+} as it enters the cell from the extracellular space, the capacitative model suggests either a chemical or physical linkage between the internal store and the plasmalemma influx pathway. A physical linkage may occur via the cytoskeleton (Rossier et al., 1991), or there may be a close apposition of ER and surface membrane proteins. In a fashion analogous to the interaction between the ryanodine-sensitive Ca^{2+}-release channel of the skeletal-muscle sarcoplasmic reticulum and the dihydropyridine receptor of the transverse tubular membrane, Irvine (1990, 1992a,b) has proposed a model in which the large cytoplasmic domain of the Ins(1,4,5)P_3 receptor interacts with the plasmalemmal Ca^{2+}-influx channel. Although clear evidence of the feet-like structures associated with the muscle triad have not been defined in electron micrographs of non-excitable cells, numerous single vesicles and complex alveolar vesicular structures in close apposition with the inner surface of the plasmalemma have been noted in vascular endothelial cells (Berne and Levy, 1988). Such features may provide the structural framework for information transfer between the lumen of the ER and the extracellular space.

Close proximity of ER and plasma membranes may also allow for either direct or indirect regulation of ER channels. Recent studies have demonstrated that Ins(1,4,5)P_3 binding or effect on the Ca^{2+}-release channel of the ER is affected by $[Ca^{2+}]_i$ in a biphasic manner; as $[Ca^{2+}]_i$ increases from the basal level, the effect of Ins(1,4,5)P_3 appears to be enhanced, whereas at higher $[Ca^{2+}]_i$ the response is attenuated (Willems et al., 1990; Iino, 1990; Pietri et al., 1990; Zhao and Muallem, 1990). Thus Ca^{2+} influx via surface membrane channels could play an important role in agonist-induced Ca^{2+} oscillations (Parker and Ivorra, 1990; Loessberg et al., 1991; Finch et al., 1991; Laskey et al., 1992; Zhang and Muallem, 1992) via both positive and negative feedback mechanisms.

The purpose of the present study was to obtain evidence for direct ionic communication between the ER and extracellular space. Toward this end we have examined the effect of the Ca^{2+}-influx blocker, SKF 96365, on unidirectional $^{45}Ca^{2+}$ efflux stimulated by bradykinin and by thapsigargin and BHQ in cultured vascular endothelial cells and human skin fibroblasts. The results suggest that Ca^{2+} influx occurs into a diffusion-limited cytoplasmic space bounded by plasmalemma on one side and junctional ER on the other. Additionally, the experiments suggest that Ca^{2+} accumulation in this restricted space can modulate Ins(1,4,5)P_3-induced Ca^{2+} release from internal stores and that most of the Ca^{2+} released via the ER Ins(1,4,5)P_3 channel enters the bulk of the cytoplasm. These results not only indicate compartmentalized cytoplasm, but also suggest that ion channels and pumps of both the plasmalemma and ER may exist in distinct membrane domains.

MATERIALS AND METHODS

Solutions and reagents

Unless otherwise indicated, Hepes-buffered saline (HBS) contained the following: 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM D-glucose, 0.1% BSA and 15 mM Na/Hepes, pH 7.4. Ca^{2+}-free HBS contained 0.3 mM EGTA and the same salts as HBS without added $CaCl_2$. Fura-2 acetoxymethyl ester (fura-2/AM) and bis-(*o*-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid acetoxymethyl ester (BAPTA/AM) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Bradykinin, saponin and thapsigargin were obtained from Calbiochem (San Diego, CA, U.S.A.), and BHQ was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals and salts were of reagent grade. SKF 96365 (hydrochloride salt) was a gift from SmithKline Beecham Pharmaceuticals (U.K.).

Culture of vascular endothelial cells

Calf pulmonary-artery endothelial cells (CPAE) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% (v/v) heat-inactivated fetal bovine serum, 1% PSN Antibiotic Mixture (Gibco, Gaitherburg, MD, U.S.A.) and 2 mM glutamine as previously described (Elliott and Schilling, 1990). For passage, cells were dispersed by trypsin treatment and seeded at a density of 3.5×10^3 cells/cm^2. Experiments were performed with cells in passages 10–17 and at 1–3 days post-confluence. The growth medium was replaced with fresh medium approx. 24 h before each experiment. All cultures demonstrated typical contact-inhibited cobblestone appearance.

Culture of human skin fibroblasts

Human skin fibroblasts, obtained from American Type Culture Collection (cell line CCD-187), were cultured in DMEM supplemented with 10% heat-inactivated fetal-bovine serum, 1% PSN Antibiotic Mixture and 2 mM glutamine. For passage, cells were dispersed by trypsin treatment and seeded at a density of 3.5×10^3 cells/cm^2. Experiments were performed with cells in passages 6–17 at 7 or 15 days post-seeding as indicated in the text. In some experiments, fibroblasts were allowed to proliferate for 6 days in complete DMEM, at which time the medium was changed to serum-free DMEM and the cells were incubated for an additional 24 h before experimentation.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by using the fluorescent indicator fura-2, as previously described (Schilling et al., 1989). Briefly, cells were enzymically dispersed, washed and resuspended in HBS containing 20 μM fura-2/AM. After incubation for 30 min at 37 °C, the cell suspension was diluted > 10-fold and incubated for an additional 30 min, washed, and resuspended. Samples from this final suspension were centrifuged and washed twice immediately before fluorescence measurement using an SLM 8000 spectrophotofluorimeter. Excitation wavelength alternated between 340 and 380 nm and the emission wavelength was 510 nm. All measurements were corrected to autofluorescence by using unloaded cells. Calibration of the fura-2 associated with the cells was accomplished by using Triton lysis in the presence of saturating bivalent-cation concentration followed by addition of EGTA (pH 8.5). $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. (1985). Test agents used had no effect on fura-2 fluorescence itself or on autofluorescence of unloaded cells when examined at the concentrations employed in this study. The Figures show representative traces from experiments performed at least three times. All fura-2 and $^{45}Ca^{2+}$-flux experiments (see below) were performed at room temperature (20–22 °C).

Measurement of $^{45}Ca^{2+}$ uptake into endothelial cell monolayers

Uptake of $^{45}Ca^{2+}$ into CPAE monolayers was performed as previously reported (Schilling et al., 1989; Elliott and Schilling, 1991). Briefly, the culture medium was removed by aspiration from confluent monolayers in 35 mm dishes and replaced with 2 ml of HBS. After a 5 min equilibration period, uptake was initiated by addition of a small amount of a solution containing $^{45}Ca^{2+}$ alone (final concn. 10–25 μCi/ml) or $^{45}Ca^{2+}$ plus bradykinin. Uptake was terminated by rapid aspiration of the reaction medium, followed by washing of the monolayer (three times) with ice-cold HBS containing 0.2 mM $LaCl_3$. Radioactivity was determined on NaOH digests of the monolayers. Where indicated, n is the number of determinations for each experimental condition performed in triplicate.

Measurement of $^{45}Ca^{2+}$ efflux from endothelial and fibroblast cell cultures

Efflux of $^{45}Ca^{2+}$ was measured as previously described (Elliott and Schilling 1991; Schilling et al., 1992). Briefly, cells grown in 35 mm culture dishes were equilibrated with radioisotope (10–25 μCi/ml) for 15–18 h. The medium was aspirated from the dish and the monolayer was immediately washed three times with HBS (without BSA). After the final wash, 3 ml of HBS was added to the dish and the cells were allowed to equilibrate for 5 min, at which time duplicate samples were withdrawn (zero time for all experiments) before the addition of test agents. Radioactivity was determined by standard liquid-scintillation technique. All values are expressed as the percentage of radioisotope remaining associated with the cell monolayer relative to the value at the end of the 5 min equilibration period. Where indicated, n is the number of determinations for each experimental condition performed in duplicate.

Loading the cells with BAPTA

In some experiments, the cells were loaded intracellularly with BAPTA. For fura-2 experiments, cells were first incubated in HBS containing 10 μM BAPTA/AM for 40 min at 37 °C. The cells were subsequently washed, resuspended and loaded with fura-2/AM as described above. For $^{45}Ca^{2+}$-efflux experiments, monolayers of cells loaded to equilibrium with $^{45}Ca^{2+}$ were incubated in complete DMEM containing 10 μM BAPTA/AM for 40 min at 37 °C. The monolayers were then treated as described above for measurement of $^{45}Ca^{2+}$ efflux.

Permeabilization of the cells with saponin

In some $^{45}Ca^{2+}$-efflux experiments, the cells were partially permeabilized with saponin. Control experiments were performed to determine the concentration of saponin that would permeabilize the cells (as judged by an increase in $^{86}Rb^+$ efflux), but would not result in detachment of the cells from the bottom of the culture dish during the efflux experiment. The optimum saponin concentration was found to be 0.001%. At this concentration permeabilization was slow, releasing $^{86}Rb^+$ at a rate of ~ 10%/min. Control $^{86}Rb^+$ efflux in the absence of saponin was ~ 0.5%/min.

RESULTS

Effect of SKF 96365 on $^{45}Ca^{2+}$ efflux

Previous studies have shown that agonist-stimulated Ca^{2+} influx is inhibited by SKF 96365 (Schilling et al., 1992). As seen in Figure 1 (upper panel), addition of bradykinin (50 nM) to CPAE suspended in zero-Ca^{2+}/EGTA buffer produced a 2–3-fold increase in $[Ca^{2+}]_i$ which returned to basal values within 2 min. These changes in $[Ca^{2+}]_i$ reflect Ca^{2+} release from internal stores and subsequent efflux from the cytosolic compartment. Re-addition of Ca^{2+} to the extracellular buffer after $[Ca^{2+}]_i$ had returned to basal value caused a large increase in $[Ca^{2+}]_i$ indicative of Ca^{2+} influx from the extracellular space. The bradykinin-induced release of Ca^{2+} from internal stores was unaffected by the presence of SKF 96365 (50 μM); however, the increase in $[Ca^{2+}]_i$ seen upon re-addition of Ca^{2+} to the extracellular buffer was significantly inhibited by this compound. Control experiments showed that 50 μM SKF 96365 was a maximally effective concentration for inhibition of the Ca^{2+}-influx component of the fura-2 response and that blockade of influx was essentially instantaneous at this concentration (results not shown). Thus SKF 96365 appears to be a selective inhibitor of receptor-initiated Ca^{2+} entry. SKF 96365 also inhibits BHQ-, cyclopiazonic acid- and thapsigargin-stimulated Ca^{2+} influx without affecting Ca^{2+} release from the internal stores (Schilling et al., 1992).

In order to test the hypothesis that the agonist-activated Ca^{2+} influx pathway allows the movement of Ca^{2+} from the extracellular space directly to the ER, we first examined the effect of SKF 96365 on unidirectional $^{45}Ca^{2+}$ efflux. If such a pathway exists, efflux should be inhibited by blockade of the influx pathway, i.e. at least part of the radiotracer efflux should occur through the open influx pathway. In the absence of agonist, $^{45}Ca^{2+}$ efflux is essentially monoexponential over the time frame examined and is unaffected by the presence of SKF 96365 (Figure 1, lower panel). Addition of a supermaximal concentration of bradykinin (50 nM) produced a dramatic increase in $^{45}Ca^{2+}$ efflux; 50–60% of the $^{45}Ca^{2+}$ associated with the cell monolayer was released within 2 min after bradykinin. In the presence of SKF 96365, the bradykinin-stimulated $^{45}Ca^{2+}$ efflux was significantly inhibited, demonstrating that, at least in part, efflux occurs via the influx pathway. The remaining efflux presumably occurs via the plasmalemmal Ca^{2+}-ATPase.

If the SKF-sensitive component of $^{45}Ca^{2+}$ efflux reflects retrograde movement of Ca^{2+} through the influx pathway, SKF 96365 should have no effect in cells that lack this pathway. As seen in

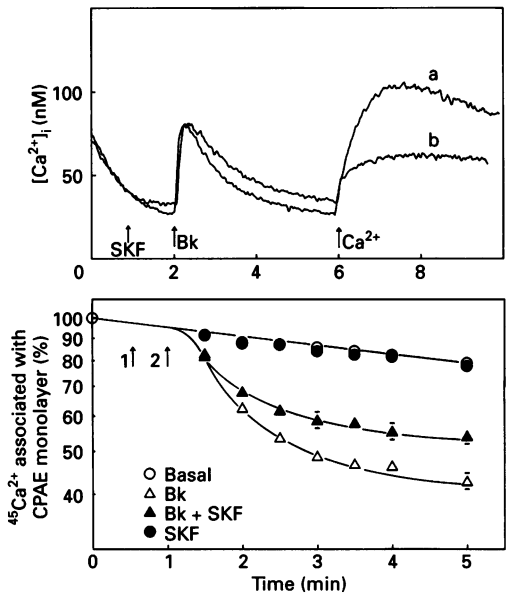

Figure 1 Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux in CPAE

Top panel: bradykinin (Bk; 50 nM) and Ca^{2+} (2 mM) were added at the times indicated by the arrows to fura-2-loaded CPAE suspended in Ca^{2+}-free HBS in the absence of SKF 96365 (trace a) or after addition of SKF 96365 (SKF; 50 μM) at the first arrow (trace b). Bottom panel: efflux of $^{45}Ca^{2+}$ was determined in CPAE monolayers as described in the Materials and methods section under basal conditions (○) and after addition of SKF 96365 (SKF) at arrow 1 (●; 50 μM), bradykinin (Bk) at arrow 2 (△; 50 nM), or SKF 96365 followed by bradykinin (▲). Values represent means ± S.E.M. for 7–10 monolayers. Where not shown, the S.E.M. was smaller than the size of the symbol employed in this and all subsequent Figures.

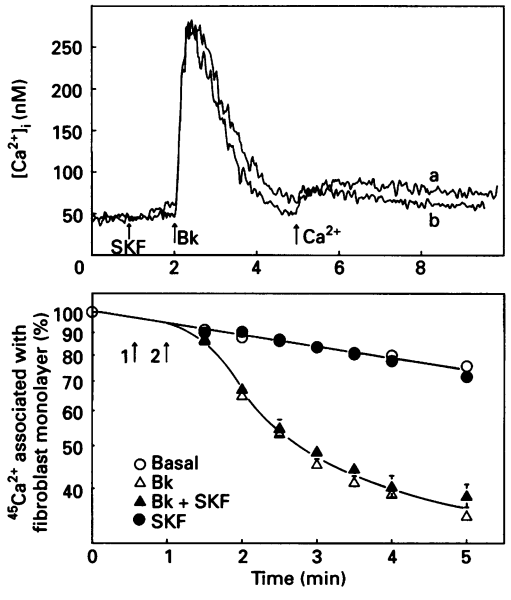

Figure 2 Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux in cultures of 7-day-old fibroblasts

Top panel: bradykinin (Bk; 50 nM) and Ca^{2+} (2 mM) were added at the times indicated by the arrows to fura-2-loaded 7-day-old fibroblasts suspended in Ca^{2+}-free HBS in the absence of SKF 96365 (trace a) or after addition of SKF 96365 (SKF; 50 μM) at the first arrow (trace b). Bottom panel: Efflux of $^{45}Ca^{2+}$ was determined in 7-day-old fibroblast cell cultures under basal conditions (○) and following addition of SKF 96365 (SKF) at arrow 1 (●; 50 μM), bradykinin (Bk) at arrow 2 (△; 50 nM), or SKF 96365 followed by bradykinin (▲). Values represent means ± S.E.M. for 7–9 monolayers.

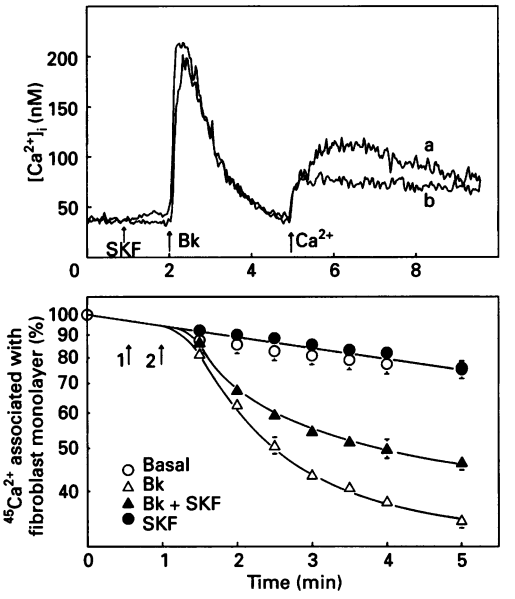

Figure 3 Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux in cultures of 15-day-old fibroblasts

Top panel: bradykinin (Bk; 50 nM) and Ca^{2+} (2 mM) were added at the times indicated by the arrows to fura-2-loaded 15-day-old fibroblasts suspended in Ca^{2+}-free HBS in the absence of SKF 96365 (trace a) or after addition of SKF 96365 (SKF; 50 μM) at the first arrow (trace b). Bottom panel: efflux of $^{45}Ca^{2+}$ was determined in cultures of 15-day-old fibroblast cell cultures under basal conditions (○) and after addition of SKF 96365 (SKF) at arrow 1 (●; 50 μM), bradykinin (Bk) at arrow 2 (△; 50 nM), or SKF 96365 followed by bradykinin (▲). Values represent means ± S.E.M. for 9 monolayers.

Figure 2 (upper panel), addition of bradykinin to human skin fibroblasts in zero-Ca^{2+}/EGTA buffer caused a transient increase in $[Ca^{2+}]_i$. Subsequent addition of Ca^{2+} produced only a small step increase in $[Ca^{2+}]_i$ back to the normal basal value. The profile obtained was unaffected by the presence of SKF 96365. Likewise, neither the basal nor the bradykinin-stimulated change in unidirectional $^{45}Ca^{2+}$ efflux was affected by SKF 96365. In these experiments, the fibroblasts were allowed to proliferate until they just covered the bottom of the dish, i.e. for 7 days post-seeding. We noticed, however, that if the cells were allowed to grow for 15 days post-seeding, an SKF 96365-sensitive Ca^{2+}-influx component was observed during measurement of $[Ca^{2+}]_i$ using fura-2. Likewise, a component of bradykinin-stimulated $^{45}Ca^{2+}$ efflux, inhibitable by SKF 96365, was seen (Figure 3). A similar profile was obtained if the cells were grown for 6 days post-seeding and serum-depleted for 18–24 h before the experiment (Figure 4). Clearly, the presence of an SKF-sensitive influx component as seen in the fura-2 experiments correlates with the inhibition of agonist-stimulated $^{45}Ca^{2+}$ efflux by SKF 96365. Furthermore, these experiments demonstrate that phenotypic expression of the Ca^{2+}-influx pathway can be reproducibly manipulated by variation in the culture conditions.

Effect of intracellular BAPTA on $^{45}Ca^{2+}$ efflux

$^{45}Ca^{2+}$ that is released from internal stores after stimulation by bradykinin may pass directly out of the endothelial cell through an agonist-activated flux pathway between the lumen of the ER and the extracellular space. Alternatively, $^{45}Ca^{2+}$ may first enter the cytoplasm and then exit from the cell. To distinguish between these possibilities, the effect of loading the cells with the Ca^{2+} chelator BAPTA was examined. If a pathway of direct

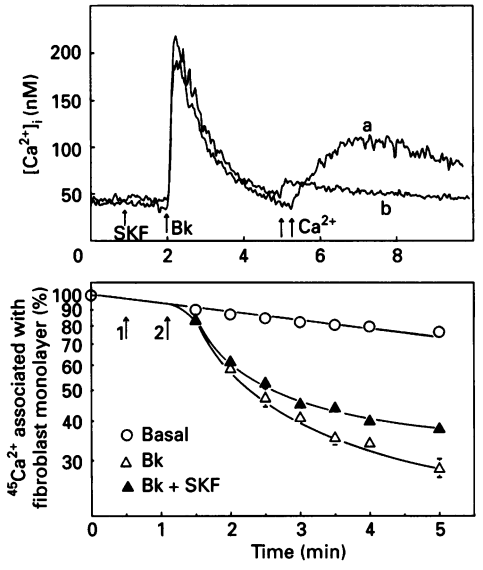

Figure 4 Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux in serum-depleted fibroblasts

Top panel; bradykinin (Bk; 50 nM) and Ca^{2+} (2 mM) were added at the times indicated by the arrows to fura-2-loaded serum-depleted fibroblasts suspended in Ca^{2+}-free HBS in the absence of SKF 96365 (trace a) or after addition of SKF 96365 (50 μM) at the first arrow (trace b). Bottom panel: efflux of $^{45}Ca^{2+}$ was determined in serum-depleted fibroblast cell cultures under basal conditions (○) and after addition of bradykinin (Bk) at arrow (△; 50 nM), or SKF 96365 (SKF) at arrow 1 followed by bradykinin at arrow 2 (▲). Values represent means ± S.E.M. for 6–9 monolayers.

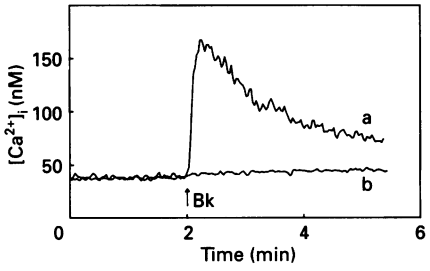

Figure 5 Effect of cytosolic BAPTA on bradykinin-induced changes in $[Ca^{2+}]_i$ of CPAE

At the time indicated by the arrow, bradykinin (Bk; 50 nM) was added to fura-2-loaded CPAE. Trace a shows the response in control cells, and trace b shows the response of CPAE loaded with BAPTA as described in the Materials and methods section.

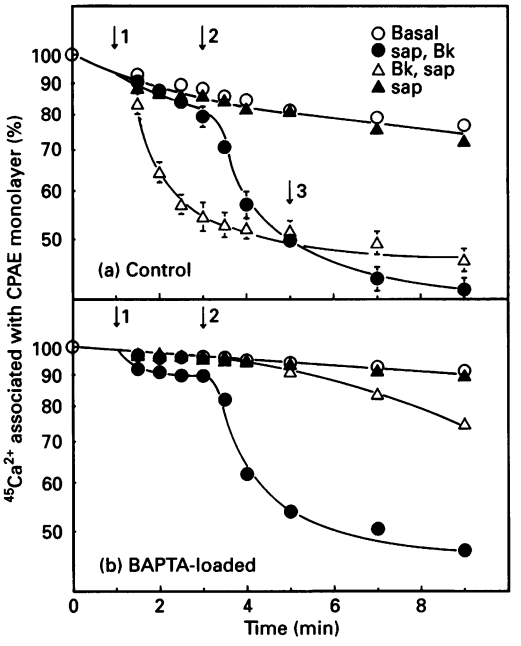

Figure 6 Effect of cytosolic BAPTA on bradykinin-induced changes in $^{45}Ca^{2+}$ efflux from CPAE monolayers

(**a**) $^{45}Ca^{2+}$ efflux from control CPAE monolayers was determined under basal conditions (○), and after addition of (1) saponin (sap) at arrow 1 (▲), (2) bradykinin (Bk) at arrow 1, followed by saponin at arrow 3 (△), and (3) saponin at arrow 1, followed by bradykinin at arrow 2 (●). (**b**) Response of BAPTA-loaded CPAE monolayers under basal conditions (○), and after addition of (1) saponin at arrow 1 (▲), (2) bradykinin at arrow 1, followed by saponin at arrow 2 (△), and (3) saponin at arrow 1 followed by bradykinin at arrow 2 (●). All values represent means ± S.E.M. for 5–10 monolayers.

communication between the ER and the extracellular space exists, there should be a component of $^{45}Ca^{2+}$ efflux that is insensitive to cytoplasmic BAPTA. However, if Ca^{2+} must first enter the cytosol before leaving the cell, the bradykinin-stimulated $^{45}Ca^{2+}$ efflux should be completely inhibited by intracellular loading of BAPTA. Addition of bradykinin to control CPAE suspended in normal HBS produced the typical biphasic increase in $[Ca^{2+}]_i$ (Figure 5). Although the basal $[Ca^{2+}]_i$ in BAPTA-loaded cells was not significantly different from control, cells loaded with BAPTA were unresponsive to addition of bradykinin. Thus intracellular BAPTA appears to prevent the rise in $[Ca^{2+}]_i$ associated with both the release of Ca^{2+} from internal stores and influx of Ca^{2+} from the extracellular space.

The comparison of $^{45}Ca^{2+}$ efflux in control and BAPTA-loaded cells is shown in Figure 6. These experiments were performed in nominally Ca^{2+}-free HBS (without EGTA). In control cells, bradykinin produced a large increase in $^{45}Ca^{2+}$ efflux (Figure 6a, △). In contrast, bradykinin had no effect on $^{45}Ca^{2+}$ efflux in BAPTA-loaded cells (Figure 6b, △).

It is possible that BAPTA may accumulate within the ER and thus inhibit bradykinin-stimulated Ca^{2+} release. To test this possibility, $^{45}Ca^{2+}$ efflux was determined in cells in which the surface membrane was selectively permeabilized with saponin. If BAPTA is present in the ER, the release of Ca^{2+} by bradykinin should be inhibited after permeabilization of the surface membrane. In control cells, saponin had no effect either on basal $^{45}Ca^{2+}$ efflux or on the ability of bradykinin to stimulate $^{45}Ca^{2+}$ efflux (Figure 6a, ▲ and ● respectively). In BAPTA-loaded cells, addition of saponin produced a small release of $^{45}Ca^{2+}$ and subsequent addition of bradykinin produced a large efflux. These results demonstrate that saponin (at the concentration employed) has no effect on bradykinin-stimulated Ca^{2+} release from internal stores and that BAPTA is excluded from the Ins(1,4,5)P_3-sensitive compartment. Another possibility is that BAPTA may inhibit one of the steps in the signal-transduction pathway and thus prevent Ca^{2+} release. If Ca^{2+} is released from internal stores in BAPTA-loaded cells, permeabilization of the cell with saponin should release the BAPTA–$^{45}Ca^{2+}$ complex when saponin is added after bradykinin. Addition of saponin after bradykinin in BAPTA-loaded cells caused an increase in $^{45}Ca^{2+}$ efflux (Figure 6b, △), consistent with the hypothesis that BAPTA itself does not inhibit Ca^{2+} release. As an additional control, the uptake of $^{45}Ca^{2+}$ was determined in the absence and presence of bradykinin

Table 1 Effect of BAPTA loading on bradykinin-stimulated $^{45}Ca^{2+}$ uptake in CPAE

A portion of $^{45}Ca^{2+}$ (10–25 μCi/dish) alone or $^{45}Ca^{2+}$ plus bradykinin (final concn. 50 nM) was added to CPAE monolayer incubated in HBS. The uptake reaction was terminated after 5 min by rapid aspiration of the buffer, followed by washing with ice-cold HBS containing 0.2 mM $LaCl_3$ as described in the Materials and methods section. Values are expressed as means ± S.E.M. ($n = 3$) fold change relative to the basal value for control non-BAPTA-loaded cells.

CPAE	Basal (fold change)	Bradykinin-stimulated (fold change)
Control	1.0	1.8 ± 0.3
BAPTA-loaded	0.85 ± 0.06	4.9 ± 0.9

in control and BAPTA-loaded cells (Table 1). Uptake of $^{45}Ca^{2+}$ was increased approx. 2-fold in control CPAE and 5-fold in BAPTA-loaded cells. These results demonstrate that intracellular BAPTA blocks $^{45}Ca^{2+}$ efflux by chelation rather than through inhibition of some step in the signal-transduction pathway and provide conclusive evidence that Ca^{2+} must first enter the cytoplasm before leaving the cell via either the Ca^{2+} pump or the SKF 96365-sensitive influx pathway.

Effect of SKF 96365 on BHQ- and thapsigargin-induced $^{45}Ca^{2+}$ efflux

As previously reported (Schilling et al., 1992), selective inhibition of the ER Ca^{2+}-ATPase/pump by BHQ or thapsigargin stimulates unidirectional efflux of $^{45}Ca^{2+}$ from CPAE monolayers (Figure 7). $^{45}Ca^{2+}$ efflux produced by these compounds was substantially slower than that seen with bradykinin. However, in contrast with the partial inhibitory effect of SKF 96365 on bradykinin-stimulated $^{45}Ca^{2+}$ efflux (Figures 1, 3 and 4), SKF 96365 completely blocked the BHQ-induced $^{45}Ca^{2+}$ efflux. Likewise thapsigargin-induced $^{45}Ca^{2+}$ efflux was substantially eliminated by SKF 96365, showing only a small increase over basal during the final 1 min of the measurement. These results suggest that essentially all of the Ca^{2+} released from the ER after pump inhibition exits from the cell through the influx pathway, i.e. $^{45}Ca^{2+}$ that 'leaks' from the ER after inhibition of the Ca^{2+} pump by thapsigargin or BHQ does not exit from the cell via the plasmalemmal Ca^{2+} ATPase/pump. However, in a fashion similar to that seen with bradykinin, both BHQ- and thapsigargin-stimulated $^{45}Ca^{2+}$ efflux was prevented by loading the cells with BAPTA, again suggesting that Ca^{2+} must first enter the cytosol before leaving the cell via the SKF 96365-sensitive influx pathway. Control experiments demonstrated that bradykinin-stimulated $^{45}Ca^{2+}$ efflux was unaffected by prior addition of BHQ or thapsigargin, confirming that these agents do not inhibit the surface membrane Ca^{2+}-ATPase/pump. These results suggest that (*a*) Ca^{2+} released from the ER enters a restricted cytoplasmic compartment that is accessible to BAPTA, but not emptied by the plasmalemmal Ca^{2+} pump, and (*b*) BHQ- and thapsigargin-sensitive Ca^{2+} pumps of the ER are in close proximity to the plasmalemmal SKF-sensitive Ca^{2+} influx pathway.

Effect of SKF 96365 on $^{45}Ca^{2+}$ efflux produced by sub-maximal concentration of bradykinin

If the differential effect of SKF 96365 on bradykinin- and BHQ-stimulated $^{45}Ca^{2+}$ efflux reflects either the kinetics or the magnitude of the Ca^{2+} released from the internal stores, then perhaps $^{45}Ca^{2+}$ efflux produced by a sub-maximal concentration of bradykinin would be inhibited to a greater extent by SKF 96365 compared with that seen with the higher concentration of bradykinin. At 2 nM bradykinin a very small increase in $^{45}Ca^{2+}$ efflux is observed relative to basal (Figure 8, upper panel).

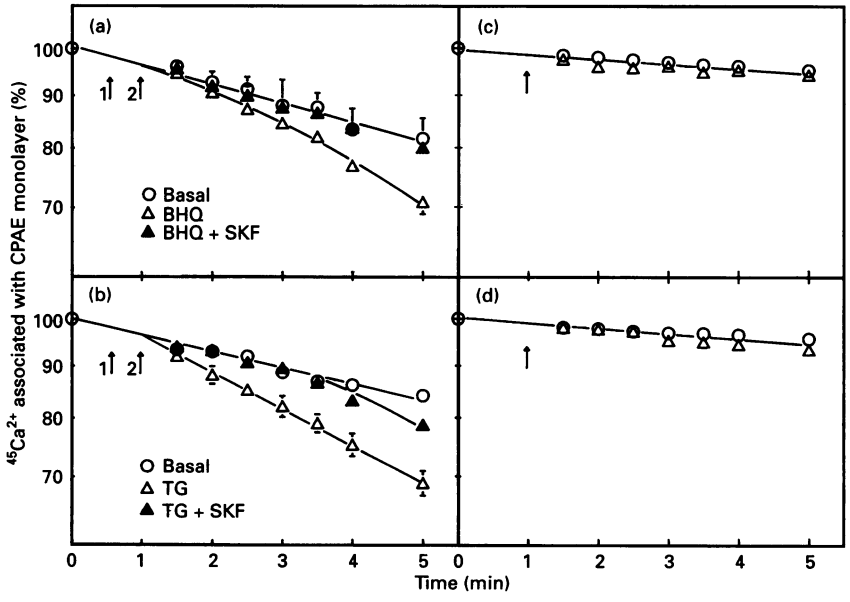

Figure 7 Effect of SKF 96365 on thapsigargin- and BHQ-induced $^{45}Ca^{2+}$ efflux from control and BAPTA-loaded CPAE

(**a**) Efflux of $^{45}Ca^{2+}$ was determined in CPAE monolayers under basal conditions (○) and after addition of BHQ at arrow 2 (△; 10 μM), or SKF 96365 (SKF; 50 μM) at arrow 1, followed by BHQ at arrow 2 (▲). (**b**) Same as in (**a**), with thapsigargin (TG; 200 nM) added at the times indicated for BHQ. (**c**) and (**d**) $^{45}Ca^{2+}$ efflux was determined in BAPTA-loaded CPAE monolayers under basal conditions (○) and after addition of either BHQ (**c**) or thapsigargin (**d**) at the arrow (△). Values represent means ± S.E.M. for 5–10 monolayers.

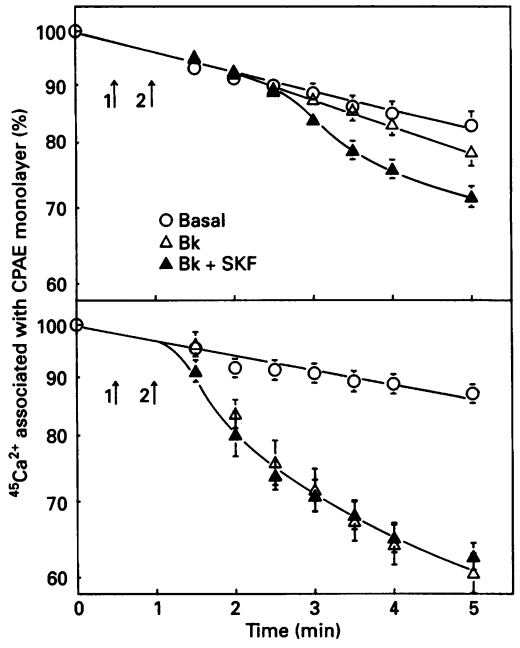

Figure 8 Effect of SKF 96365 on $^{45}Ca^{2+}$ efflux produced by sub-maximal concentrations of bradykinin in CPAE monolayers

Upper panel: efflux of $^{45}Ca^{2+}$ was determined in CPAE monolayers as described in the Materials and methods section under basal conditions (○) and after addition of bradykinin (Bk; 2 nM) at arrow 2 (△), or SKF 96365 (SKF; 50 μM) at arrow 1 followed by bradykinin at arrow 2 (▲). Lower panel: same as in upper panel, with 3 nM bradykinin added at arrow 2. Values represent means ± S.E.M. for 5 monolayers.

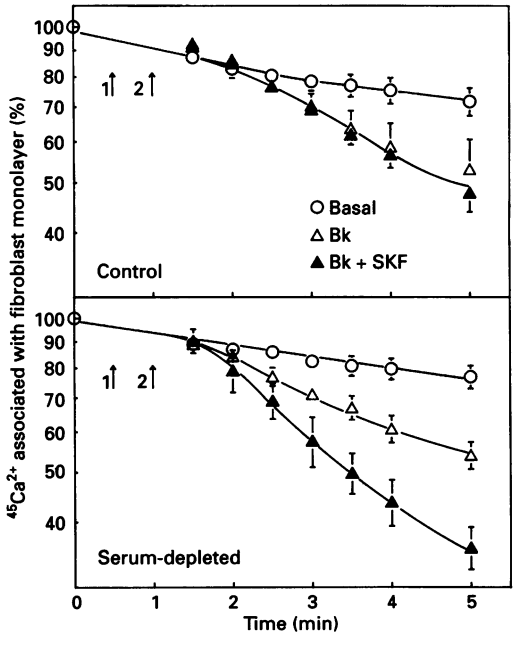

Figure 9 Effect of SKF 96365 on $^{45}Ca^{2+}$ efflux produced by sub-maximal concentrations of bradykinin in fibroblast cell cultures

Upper panel: efflux of $^{45}Ca^{2+}$ was determined in control fibroblast cell cultures as described in the Materials and methods section under basal conditions (○) and after addition of bradykinin (Bk; 3 nM) at arrow 2 (△), or SKF 96365 (SKF; 50 μM) at arrow 1, followed by bradykinin at arrow 2 (▲). Lower panel: same as in upper panel, with 1 nM bradykinin added to serum-depleted fibroblast cell cultures. Values represent means ± S.E.M. for 6 monolayers.

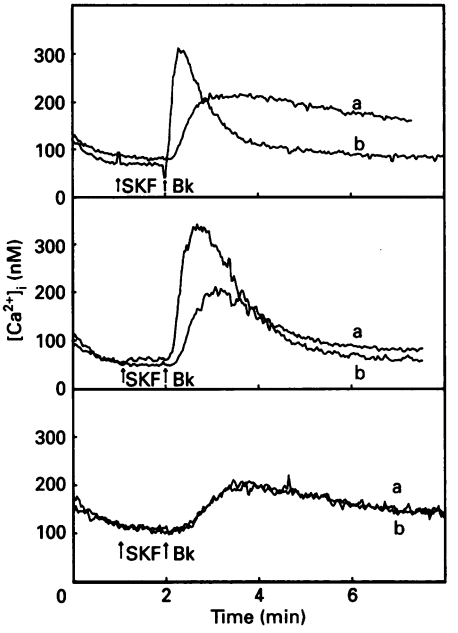

Figure 10 Effect of SKF 96365 on bradykinin-induced changes in $[Ca^{2+}]_i$ in CPAE and serum-depleted fibroblasts

Upper panel: bradykinin (Bk; 2 nM) was added at the times indicated by the arrows to fura-2-loaded CPAE suspended in normal HBS in the absence of SKF 96365 (trace a) or after addition of SKF 96365 (SKF; 50 μM) at the first arrow (trace b). Middle panel: same as in the upper panel, but with serum-depleted fibroblasts. Bottom panel: same as in the upper panel, but with control fibroblasts.

However, addition of 2 nM bradykinin in the presence of SKF 96365 actually produced a stimulation in $^{45}Ca^{2+}$ efflux compared with that seen in the absence of SKF 96365. At an intermediate concentration of bradykinin (3 nM), $^{45}Ca^{2+}$ efflux was unaffected by SKF 96365 (Figure 8, lower panel). Thus the effect of SKF 96365 on $^{45}Ca^{2+}$ efflux is dependent on the concentration of bradykinin.

To determine if the stimulatory effect of SKF 96365 on $^{45}Ca^{2+}$ efflux induced by a threshold concentration of bradykinin reflects blockade of the agonist-stimulated Ca^{2+}-influx pathway, experiments were repeated in control and serum-depleted fibroblasts, i.e. in the absence or presence of phenotypic expression of the influx pathway. As seen in Figure 9, SKF 96365 produced a substantial stimulation of bradykinin-induced $^{45}Ca^{2+}$ efflux in serum-depleted fibroblasts, but has no effect on $^{45}Ca^{2+}$ efflux in control fibroblasts. Thus the stimulatory effect of SKF 96365 on efflux correlates with the expression of the agonist-stimulated Ca^{2+}-influx pathway.

The above results suggest that blockade of Ca^{2+} influx increases Ca^{2+} release from internal stores during stimulation by low concentrations of bradykinin. To test this hypothesis directly, the effect of SKF 96365 on $[Ca^{2+}]_i$ was examined (Figure 10). Addition of bradykinin (2 nM) to CPAE suspended in normal HBS produced a slow 2-fold increase in $[Ca^{2+}]_i$ that reached a peak within 1–2 min and remained elevated for several minutes (Figure 10, upper panel). Addition of bradykinin after SKF 96365 produced a rapid and transient 3-fold increase in $[Ca^{2+}]_i$ that reached a peak within seconds of bradykinin addition and returned to basal values within 2 min. A similar result was obtained in serum-depleted fibroblasts (Figure 10, middle panel). These results clearly demonstrate the two effects of SKF 96365. The transient component of the $[Ca^{2+}]_i$ response to low concen-

trations of bradykinin, which reflects release from internal stores, is enhanced in the presence of SKF 96365, whereas the sustained component, which reflects influx of Ca^{2+}, is blocked. In control fibroblasts that do not express the influx pathway, SKF 96365 had no significant effect on the change in $[Ca^{2+}]_i$ produced by sub-maximal concentrations of bradykinin (Figure 10, bottom panel). These results support the conclusion that SKF 96365 increases agonist-induced Ca^{2+} release from internal stores by blocking influx of Ca^{2+} from the extracellular space. Since blockade of Ca^{2+} influx by SKF 96365 is expected to decrease $[Ca^{2+}]_i$ in the immediate vicinity of the influx pathway, the above results suggest that a rise in $[Ca^{2+}]_i$ in the restricted space between the SKF-96365-sensitive influx pathway and the thapsigargin-sensitive Ca^{2+} pumps of the ER must inhibit Ins(1,4,5)P_3-induced Ca^{2+} release from the ER.

DISCUSSION

Does Ca^{2+} enter the ER directly from the extracellular space?

Although it was originally proposed that the ER may load with Ca^{2+} that is derived directly from the extracellular space, a number of recent studies suggest that Ca^{2+} must first enter the cytoplasm before it can be sequestered by the ER. Addition of thapsigargin to pancreatic acinar cells caused an increase in $[Ca^{2+}]_i$ to a steady elevated phase that was unaffected by subsequent addition of receptor agonist (Takemura et al., 1989). In vascular endothelial cells, however, it is clear that some increase in the steady-state level of $[Ca^{2+}]_i$ could be obtained upon addition of bradykinin after either thapsigargin or cyclopiazonic acid (Schilling et al., 1992). In another set of experiments it was demonstrated that the influx pathway conducts both Ca^{2+} and Ba^{2+}, but only Ca^{2+} appears to accumulate in the ER, suggesting that Ba^{2+} is not a substrate for the ER Ca^{2+}-ATPase/pump (Schilling et al., 1989). Thus Ba^{2+} accumulation and release from the ER would be consistent with a direct pathway between the extracellular space and the lumen of the ER. Kwan and Putney (1990) demonstrated that Ba^{2+} could not be released by receptor agonists in pancreatic acinar cells depleted of intracellular Ca^{2+} and subsequently loaded with Ba^{2+}. A similar result was obtained in vascular endothelial cells (Schilling and Rajan, 1990). In contrast, there is evidence in platelets that Ba^{2+} can enter and be released from the ER by thrombin (Ozaki et al., 1992). These experiments are difficult to interpret, since the high concentrations of Ba^{2+} employed in these fura-2 experiments may inhibit signal transduction. Preliminary studies in vascular endothelial cells have shown that $^{133}Ba^{2+}$ accumulates to approximately the same extent as $^{45}Ca^{2+}$, and that unidirectional $^{133}Ba^{2+}$ efflux is increased upon stimulation with receptor agonists (Schilling and Rajan, 1990). These results suggest the possibility that a direct pathway for Ba^{2+} (i.e., Ca^{2+}) movement from the extracellular space to the ER may exist in some cell types. In order to test this hypothesis, we first examined $^{45}Ca^{2+}$ efflux in the presence and absence of SKF 96365. The results of these experiments provide strong evidence that (1) SKF 96365 specifically and selectively blocks Ca^{2+} influx without effects on other steps in the signal-transduction process and (2) a component of $^{45}Ca^{2+}$ efflux exits from the cell via the influx pathway, i.e. retrograde efflux of radioisotope. Because these experiments were performed in the presence of extracellular Ca^{2+}, it could be argued that entry of non-radioactive Ca^{2+} during stimulation with agonist will change the specific radioactivity of the $^{45}Ca^{2+}$ released from ER into the cytosol and thus inhibit $^{45}Ca^{2+}$ efflux. In this case, however, blockade of influx by SKF 96365 would stimulate rather than inhibit efflux. Furthermore, bradykinin-stimulated $^{45}Ca^{2+}$ efflux in nominally Ca^{2+}-free buffer (Figure 6) is essentially identical with that obtained in Ca^{2+}-containing HBS. It is also possible that when $^{45}Ca^{2+}$ leaves the cell via the surface membrane Ca^{2+}-ATPase/pump it may move into an unstirred layer at the extracellular surface and subsequently re-enter the cell through the influx pathway. Again, however, blockade of the influx pathway by SKF 96365 would be expected to stimulate efflux rather than inhibit. Together, the results support the view that inhibition of $^{45}Ca^{2+}$ efflux by SKF 96365 reflects blockade of radioisotope efflux through the influx pathway.

To determine if Ca^{2+} moves directly from the ER to the extracellular space, we examined $^{45}Ca^{2+}$ efflux in BAPTA-loaded cells. The change in $[Ca^{2+}]_i$ seen in control CPAE upon addition of bradykinin was completely eliminated in BAPTA-loaded cells. Likewise, agonist-stimulated $^{45}Ca^{2+}$ efflux was completely inhibited by intracellular BAPTA. The blockade of efflux did not reflect BAPTA accumulation in the ER or an inhibitory effect of BAPTA on one of the steps in the signal-transduction process. These results suggest that Ca^{2+} must first enter the cytosol before leaving the cell. In pancreatic acinar cells, large increases in $^{45}Ca^{2+}$ efflux were reported upon addition of agonists to BAPTA-loaded cells (Muallem et al., 1990; Zhang and Muallem, 1992). The reason for the difference from the present study is unknown, but it may be related to the extent of BAPTA loading and/or esterase activity in acinar cells compared with CPAE. Alternatively, BAPTA may be present in a different cytosolic compartment in acinar cells.

Evidence for a diffusion-limited cytosolic compartment

In contrast with the partial inhibition of $^{45}Ca^{2+}$ efflux by SKF 96365 observed at high concentrations of bradykinin, blockade of the influx pathway produced essentially complete inhibition of $^{45}Ca^{2+}$ efflux stimulated by thapsigargin and BHQ. These results suggest that the Ca^{2+} that leaks from the ER after inhibition of the pump by these compounds does not have access to the surface membrane Ca^{2+}-ATPase/pump, but is released to a site which is near the influx pathway. This conclusion is based on the fact that these compounds are selective for the ER Ca^{2+}-ATPase and have no inhibitory effect on the surface membrane Ca^{2+} pump. This assumption is supported by the observation in the present study that neither BHQ nor thapsigargin inhibited bradykinin-stimulated $^{45}Ca^{2+}$ efflux. Furthermore, the specificity of these compounds for ER Ca^{2+}-ATPase/pump has been established in a number of other cell types (Foder et al., 1989; Moore et al., 1987). Thus there appears to be a restricted cytosolic compartment between the thapsigargin-sensitive ER and the SKF 96365-sensitive plasmalemma Ca^{2+}-flux pathway. In a recent study of BHQ-activated Ca^{2+} currents in endothelial cells, Vaca and Kunze (1993) show that depletion and refilling of the intracellular Ca^{2+} stores produces oscillations in membrane current. These oscillations occur with BAPTA in the pipette solution, but not when the cells are loaded with BAPTA via the acetoxymethyl ester form. These results are consistent with Ca^{2+}-channel flux into a restricted space that is in close association with the BHQ-sensitive Ca^{2+} pumps of the ER and suggest that the restricted space is membrane-delimited.

The presence of a limited diffusion space within the cytoplasm could have important physiological consequences. Recent studies in human sweat-gland epithelia show that, although thapsigargin produces large increases in $[Ca^{2+}]_i$ comparable with that observed with agonists, it fails to increase trans-epithelial ion transport (Pickles and Cuthbert, 1992) consistent with compartmentalized cytosolic Ca^{2+}. By analogy, thapsigargin may have little effect on NO release from endothelial cells. Compartmentalization of

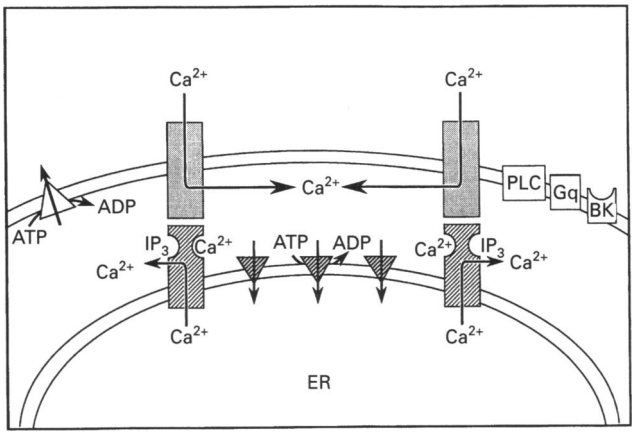

Figure 11 Vectorial Ca^{2+}-flux model

Depicted is an area of close apposition between the endoplasmic reticulum (ER), which contains the thapsigargin- and BHQ-sensitive Ca^{2+}-ATPase/pumps (shaded triangles) and the Ins(1,4,5)P_3 (IP_3) receptor (shaded rectangles), and the plasmalemma, which contains the Ca^{2+}-influx pathway (shaded rectangles) and Ca^{2+}-ATPase/pumps (open triangle). The bradykinin receptor (BK), GTP-binding protein (Gq), and phospholipase C (PLC) are also shown associated with the plasmalemma. See the text for details.

cytosolic Ca^{2+} may also be responsible for the differential effect of some agonists on NO and prostacyclin release (White and Martin, 1989; Mitchell et al., 1992).

Regulation of Ins(1,4,5)P_3-induced Ca^{2+} release by Ca^{2+} influx

There is now substantial evidence that cytosolic Ca^{2+} controls Ins(1,4,5)P_3-induced Ca^{2+} release from internal stores (Chueh and Gill, 1986; Parker and Ivorra, 1990; Willems et al., 1990; Zhao and Muallem, 1990; Finch et al., 1991). The effect of Ca^{2+} appears to be biphasic; between 0 and 200–300 nM Ca^{2+}, Ins(1,4,5)P_3-induced Ca^{2+} release is stimulated, whereas above this concentration range the effect of Ins(1,4,5)P_3 is attenuated (Iino, 1990). The mechanism by which Ca^{2+} regulates Ins(1,4,5)P_3-induced Ca^{2+} release is unknown. Ca^{2+} may alter Ins(1,4,5)P_3 binding or effect. In permeabilized hepatocytes and liver plasmalemma-enriched preparations, increasing Ca^{2+} from 0.1 to 700 nM was associated with an increase in affinity of Ins(1,4,5)P_3 with the low-affinity state apparently coupled to Ca^{2+} release (Pietri et al., 1990). At the single-channel level, the effect of Ca^{2+} at a constant Ins(1,4,5)P_3 concentration appears to be on channel open probability (P_o); the P_o was approx. 0.1 at 10 nM and 2 μM Ca^{2+}, and was maximum at 200 nM Ca^{2+} (Bezprozvanny et al., 1991). The effect of Ca^{2+} may not be direct. In cerebellum a second protein, calmedin, appears to mediate the effect of Ca^{2+} at the Ins(1,4,5)P_3 receptor (Danoff et al., 1988). In the present study, blockade of Ca^{2+} influx by SKF 96365 was associated with a large increase both in $^{45}Ca^{2+}$ efflux and in the fura-2 transient produced by sub-maximal concentration of bradykinin. This effect of SKF 96365 was seen in CPAE and in serum-depleted fibroblasts, but not in control fibroblasts, suggesting that blockade of Ca^{2+} influx is responsible for the stimulation observed. Thus Ca^{2+} influx through the SKF 96365-sensitive pathway must produce inhibition of Ca^{2+} release from internal stores. If in fact a rise in Ca^{2+} near the Ins(1,4,5)P_3 channel inhibits Ca^{2+} release, it seems paradoxical that we were able to observe stimulation, i.e. why does the Ca^{2+} released from the ER not inhibit further release? One possible explanation is that the Ca^{2+} released from the ER enters the bulk of the cytoplasm and has limited access to the restricted space subjacent to the plasmalemmal influx pathway. Directional Ca^{2+} release is supported by the finding that the stimulated efflux of $^{45}Ca^{2+}$ observed in the presence of SKF 96365 must exit from the cell through the surface membrane Ca^{2+}-ATPase/pump. The inhibition of Ca^{2+} release by Ca^{2+} influx should play an important role in agonist-induced oscillations. In support of this hypothesis, it has recently been shown in bovine aortic endothelial cells that synchronized oscillations in $[Ca^{2+}]_i$ observed in K^+-free extracellular buffer could be blocked by La^{3+} and by SKF 96365 (Laskey et al., 1992).

Model for vectorial cellular Ca^{2+} flux

A model which can explain the results of the present study is shown in Figure 11. There is a restricted cytoplasmic subplasmalemmal space (loading compartment), which exhibits limited diffusion with the rest of the cytosol and is defined by an area of surface membrane which contains the Ca^{2+}-influx pathway but is devoid of Ca^{2+} pumps (influx surface), and by a section of junctional ER that is rich in thapsigargin-sensitive Ca^{2+}-ATPase pump units (loading surface). We propose the following sequence during agonist stimulation. Ca^{2+} (1) enters the cell at the influx surface, (2) is taken up into the ER at the loading surface, (3) is released through the Ins(1,4,5)P_3 receptor to the bulk of the cytosol (release surface), and (4) exits the cells via the plasmalemma Ca^{2+} pump (efflux surface). Blockade of Ca^{2+} influx through the agonist-activated pathway of SKF 96365 increases Ca^{2+} release from internal stores and suggests that a rise in $[Ca^{2+}]_i$ within the loading compartment as a result of influx directly inhibits either Ins(1,4,5)P_3 binding or the Ins(1,4,5)P_3 effect at the Ca^{2+}-release channel. The apparent functional absence of thapsigargin-sensitive Ca^{2+} pumps at the release surface and plasmalemma Ca^{2+} pumps at the influx surface provides the structural basis for vectorial Ca^{2+} flux through the cell. A temporal and spatial separation between Ca^{2+} uptake and release sites has been proposed in parotid acinar cells (Menniti et al, 1991). Although it is clear that Ca^{2+} must be actively pumped from the cytosol into the ER, the functional consequences of the proposed model would be equivalent to the original capacitative Ca^{2+} entry model, which suggested a direct path between the extracellular space and the ER lumen. The size of the restricted cytoplasmic space is unknown, and although the model, as drawn, implies that the ER and plasmalemma are within molecular distance at the area of Ca^{2+} influx, this need not be the case. Close apposition of the two membranes, however, would allow for direct coupling between the Ins(1,4,5)P_3-release channel and the SKF 96365-sensitive influx pathway. A change in ER luminal Ca^{2+} or perhaps a change in the membrane potential of the ER upon Ca^{2+} release could induce a conformational change in the Ins(1,4,5)P_3 receptor that is then communicated to the surface membrane Ca^{2+}-influx pathway in a fashion analogous to electromechanical coupling proposed at the skeletal-muscle triad. A similar structural arrangement has been proposed in *Drosophila* photoreceptors, where the transient receptor potential (*trp*) gene codes for a plasmalemmal Ca^{2+} channel (Hardie and Minke, 1992; Minke and Selinger, 1992). The activation of this channel appears to be associated with release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive intracellular store of the sub-microvillar cisternae.

Regulation of functional expression

Despite the fact that all non-excitable cell types examined to date exhibit agonist-induced Ca^{2+} release from internal stores, the SKF 96365-sensitive Ca^{2+} influx pathway is observed in contact-inhibited CPAE and in serum-depleted fibroblasts, but not in

proliferating fibroblasts. These results suggest that phenotypic expression of the influx pathway is independent of Ins(1,4,5)P_3-receptor expression. A similar result has been reported in HeLa cells, where it was found that coupling between the depletion of the internal store and Ca^{2+} influx was absent from mitotic cells, but not from interphase cells (Preston et al., 1991). The regulation of expression may be at the translational or transcriptional levels, or expression of functional channels may reflect a structural change, i.e. the channel protein may be present but non-functional because of its location or improper alignment between the ER and plasmalemma. Although these questions cannot be addressed at present, a subtracted cDNA library obtained from proliferating and serum-depleted fibroblasts may provide a useful source for cloning the agonist-activated influx pathway.

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